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THE EXPERIMENTAL PRODUCTION OF WATERY VACUOLATION OF THE LIVER

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In this paper it will be shown that when the oxygen supply to the liver is seriously impaired, water passes from the blood into the liver cells and forms large watery vacuoles in the cell cytoplasm. This phenomenon only occurs provided that, during the period of liver anoxia, the blood pressure in the liver sinusoids is maintained at least at its normal value; if the pressure is above normal, the liver-cell vacuolation is more rapid and greater in extent. It seems that anoxia, by increasing the permeability of either the sinusoids or the liver-cell walls, allows the existing hydrostatic pressure in the sinusoids to force water, and probably other plasma constituents, into the liver cells.

It is necessary to distinguish two sets of circumstances under which this liver-cell vacuolation has been found to occur.

(i) *In vivo vacuolation.* Vacuoles may develop during life in any conditions of severe anoxia. The anoxia must be very severe, to the point of threatening life, and must persist for 20–30 min. before significant vacuolation occurs.

(ii) *Post-mortem vacuolation.* When an animal dies from anoxia (or asphyxia) vacuoles rapidly develop during the first 5–15 min. post-mortem, provided the animal is not bled out. In such an animal the venous blood pressure rises considerably just before death and a positive venous pressure persists for some time after death; this pressure is communicated back to the liver sinusoids and is sufficient to cause vacuolation of the highly anoxic post-mortem liver. In death from other causes, the intrahepatic blood pressure after death is approximately zero and so no vacuoles develop.

One proviso must be made with regard to the above statements—the liver must contain no more than a moderate amount of glycogen. If the liver is full of glycogen vacuolation will usually not occur under the conditions specified.

HISTOLOGICAL DESCRIPTION

This description applies to the rat, rabbit, guinea-pig and monkey. The vacuoles referred to are present in the liver-cell cytoplasm. In size they range from 2 to 12 μ; most commonly they are about the size of the nucleus or rather less (Pl. 1, figs. 6–11). The number in each cell ranges from 1 to about 12, most

commonly it is 2 or 3. While in general their shape is spherical, many of them are definitely 'off round', being ovoid or slightly polyhedral or flattened on one side (Pl. 1, figs. 10, 11). When there are only a few vacuoles in the cell they tend to be located centrally, close to the nucleus. Often the nucleus indents the vacuole which then appears as a crescent closely applied to one side of the nucleus. Less commonly the vacuole may indent the nucleus and the nucleus appears as a crescent applied to the vacuole. This suggests that the intravacuolar pressure is very variable. Occasionally a small vacuole has been seen lying wholly within the nucleus.

The vacuoles are clearly visible in fresh living liver cells mounted in heparinized plasma from the same animal. In the living cell the cytoplasm appears granular and usually contains some small highly refractile fat droplets. The nucleus appears as a pale grey homogeneous area bounded by a membrane and containing a nucleolus. The vacuoles appear as pale grey non-granular areas in the cytoplasm with smooth regular margins. They look very like the nucleus, and, when of comparable size, can only be distinguished by the absence of a nucleolus. If the cells are ruptured by gentle pressure, the granular cytoplasm may break away in fragments leaving the vacuoles as discrete bodies floating freely in the plasma and the vacuoles are then seen to be bounded by a definite membrane. They have no power of segregating neutral red, methylene blue or methyl green. Their optical properties (translucence, refraction) indicate definitely that they are, in fact, watery vacuoles; they are certainly neither gaseous nor lipoid, and their appearance in paraffin sections rules out any question of their being solid. The slightly irregular shape of the vacuoles, already referred to, is equally apparent in the living cell, and so it does not represent a fixation artifact. Nor is it due to mutual pressure of adjacent vacuoles, for a solitary vacuole lying well away from the nucleus may also be irregular in shape. This is interesting as it suggests the presence of an invisible structural architecture in the cell cytoplasm.

In paraffin or frozen sections of fixed liver, the vacuoles usually appear as optically empty spaces containing no stainable material. In a few cases, however, most of the vacuoles have contained a homogeneous material which stains faintly with ordinary cytoplasmic stains such as eosin. The wall of the vacuole appears smooth, sharp and clear cut. It stains a little darker than the surrounding cytoplasm and presumably represents a separation membrane between the watery contents and the surrounding colloidal cytoplasm.

Although the vacuoles are visible after any of the ordinary methods of fixation, it is best to avoid alcoholic fixatives because these precipitate the cytoplasm in the form of a very open meshwork which may present a rather vacuolated appearance. It is always possible to distinguish between the sharply demarcated true vacuoles and the more indefinite vacuolation of improperly fixed cytoplasm, but in the presence of the latter the search for true vacuoles is more tedious. Formol is not ideal, it precipitates the cytoplasm in coarse lumps and does not harden the tissue adequately for paraffin embedding. In our experience the best cytoplasmic fixative for the liver is a mixture of

formol and a metallic salt, as has been used for fixing the Golgi apparatus. Salts of uranium, cobalt and cadmium have been used for this purpose by various workers. We find that copper is equally good, and a mixture of 7 parts 1% copper sulphate and 1 part unneutralized formol has been used for most of this work.

The relationship of the various stainable cytoplasmic components to the vacuoles was investigated by the standard histological techniques. Fat was stained with Sudan IV in frozen sections of formol fixed material by the method of Kay & Whitehead (1935), and also with osmic acid. The vacuoles gave no staining reactions for fat; they were therefore not fat globules (Pl. 1, fig. 12). The cells usually contained a variable number of minute fat droplets; occasionally a few larger ones, about the size of the smallest vacuoles, were present. Once or twice, quite large globules were encountered, but these livers were probably pathological. In paraffin sections, of course, any sizable fat droplets will be represented by empty spaces which will be hardly distinguishable from the watery vacuoles under discussion. It is always essential, therefore, to examine control frozen sections stained with Sudan IV before assuming that the spaces seen in paraffin sections are true watery vacuoles. In practice it was found that the minute fat droplets are too small to appear as visible spaces in paraffin sections, and it was quite uncommon, in the species studied, to encounter any fat spaces at all. Nevertheless, the precaution of fat staining has always been adhered to because of the occasional fatty liver that may be encountered. In the few livers which contained both large fat globules and watery vacuoles, the two were often in close contact. Usually the fat globules were indented by the vacuoles, the fat appearing as a crescent applied to one side of the vacuole; sometimes a thin rim of fat extended round the whole vacuole, which then appeared to lie wholly within the fat globule. Less frequently the watery vacuoles were somewhat indented by adjacent fat globules.

Glycogen was stained by Best's carmine, after Carnoy fixation (Pl. 1, fig. 13); mitochondria by iron haematoxylin or Altmann's acid fuchsin, after Regaud fixation; cytoplasmic ribonucleic acid by toluidin blue after Carnoy fixation (Davidson & Waymouth, 1944). No vacuolar content could be stained by these methods. The glycogen, mitochondria and ribonucleic acid aggregates showed no particular orientation to the vacuoles, nor was there any change in these components as compared with normal cells. When ordinary watery fixatives are employed, the glycogen, after being precipitated in irregular masses, is dissolved out in the water, leaving irregular empty spaces in the cytoplasm. In the case of cells rich in glycogen these 'glycogen spaces' may be quite large, and this normal appearance of glycogen-rich liver cells has sometimes been referred to, incorrectly, as 'vacuolation'. The glycogen spaces are not vacuoles, nor can they be confused with vacuoles, for they are irregular in shape, have no bounding membrane and usually contain a sparse reticulum of cytoplasm. Attempts to stain the Golgi apparatus were unsuccessful. The bile canaliculi were sometimes clearly seen in preparations stained with iron haemotoxylin or Hollande's chlorocarmine, after Carnoy fixation; they were also seen in fresh fragments of liver mounted in plasma. No connexion could be traced between canaliculi and vacuoles; there was nothing to suggest that the vacuoles were distended intracellular bile canaliculi. The 'dark' and 'light' liver cells described by Scharrer (1938) were seen with iron haematoxylin after Bouin fixation, and vacuoles were present equally in both types.

As already mentioned, the vacuoles sometimes contained a homogeneous material which stained lightly with cytoplasmic stains. This 'content' stained a very pale red with eosin, grey with iron haematoxylin, pale bluish grey with Romanowsky stains, and usually blue with the haemalum-aurantia-aniline blue method of Marshall & Trowell (1943). This latter method stains the cytoplasm yellowish-brown and so affords a good differential stain for the vacuolar content. The content is better preserved by formol-copper or formol-bichromate fixatives than by formol alone. It seems unlikely that this content can be anything other than protein, and in appearance and staining reactions it exactly resembles the plasma protein which may be seen in adjacent blood vessels.

The histological conclusion is that the vacuoles consist of a watery fluid which contains a variable amount of protein, possibly plasma protein.

PRELIMINARY OBSERVATIONS

This liver vacuolation was first seen in 1940 during a routine study of histological changes produced in animals by rapid 'decompression'. In these experiments, performed by Eggleton, Elsden, Fegler & Hebb (1945), groups of animals were rapidly decompressed from 1·0 to 0·16 atm. in an atmosphere approximating to pure oxygen. After 2 hr. at the low pressure, by which time some had died, the animals were returned to atmospheric pressure and the survivors killed with coal gas. Autopsies were made at times ranging from 5 min. to 2 hr. after death, and liver samples, examined histologically, showed vacuoles in some, but not all, of these animals. Vacuoles were found both in those which had died as a result of decompression and those which had survived. Vacuoles were produced in this way in the rat, rabbit, guinea-pig, goat and monkey, and these findings have already been reported (Trowell, 1943).

During the war, German workers also discovered these vacuoles. Mueller & Rotter (1942) found them in four airmen who died from the effects of high altitude. Pichotka (1942) found them in guinea-pigs after exposure to an atmosphere of reduced oxygen content at normal pressure. Hesse (1942) found them in humans killed by acute asphyxia (suffocation, drowning). Ladewig (1943) summarized this work and regarded it as proved that the vacuoles were always the result of anoxia occurring during life and believed that vacuolation could be used as a histological criterion for the occurrence of liver anoxia.

In the decompression experiments referred to above, although oxygen was passed through the chamber throughout the experiment, analysis showed that, after decompression, the oxygen partial pressure might be only 80–90 mm. Hg. The animals were therefore subject to some degree of anoxia, and in the light of the German work it seemed that the vacuolation should be attributed to this cause. Certain additional facts were discovered, however, which did not seem to fit in with the anoxia theory. First, since the oxygen tension is presumably least at the centre of the liver lobule, vacuoles might be expected to occur there first; but this was usually not the case. In fifty-three rabbits examined, vacuolation was confined to the centre of the lobules in only three cases; in the others the distribution was midzonal (25), peripheral (18) and throughout the lobule (5). Secondly, in further experiments (carried out variously by J. Fegler, A. W. Missiuro and C. O. Hebb) groups of rabbits were decompressed to various pressures in atmospheres of known oxygen content, and analysis of 200 animals from these experiments showed that the proportion of animals showing vacuoles could be correlated neither with the partial pressure of oxygen to which they had been exposed (range 24–53 mm. Hg) nor with the time for which they had been exposed (range 5–87 min.). Thirdly, vacuoles were found in animals which had been decompressed from 6·0 to

1·0 atm. and had died instantaneously as a result; in this case the question of anoxia seemed to be absolutely excluded.

Histological examination of all the livers obtained from decompression experiments yielded two clues which served as a starting point for further investigation. First, when the livers were classified into three groups on the basis of their glycogen content (histologically assessed), it was found that vacuolation occurred most frequently in the glycogen-poor livers and least frequently in the glycogen-rich livers. This is shown in the case of the rabbit in Table 1. Secondly, it was noticed in all species that the vacuolated livers were always greatly congested with blood.

TABLE 1. The relation between incidence of vacuolation and liver glycogen content in 200 rabbits dying in decompression experiments

	Total	Vacuolated	% vacuolated
Livers poor in glycogen	58	39	67
Livers moderate in glycogen	90	21	23
Livers rich in glycogen	52	3	6
All livers	200	63	32

It seemed probable, therefore, that anoxia was not the only factor concerned in vacuole formation, and the problem was now investigated by direct experiment.

METHODS

Experiments were performed on adult rats. The rats were starved for 24 hr. before the experiment, in order to avoid high liver-glycogen levels, which, as will be shown later, may prevent vacuolation in conditions under which it would otherwise occur. When anaesthesia was employed, it was necessary to guard against the risk of anoxia resulting from respiratory depression; for this reason 'open ether' was always used. In anaesthetized animals and also with isolated livers it was possible to remove liver samples at intervals during the experiment. The rat's liver has seven accessible lobes, and the distal part of each can be cut off, the stump being previously ligated with string. Using wet string, 1 mm. in diameter, a single twist of a knot tightened gently was sufficient to secure haemostasis without cutting through. After fixation in formol-copper sulphate for 48 hr., each sample was cut in half. Paraffin sections were made from one half and, if these showed vacuoles, then frozen sections stained with Sudan IV were made from the other half to exclude the possibility of fat. Control experiments showed that prolonged ether anaesthesia did not itself produce vacuoles, and that when vacuoles were produced experimentally, their extent and distribution was substantially the same in the different lobes.

RESULTS

(1) *Anoxic death*

The claim of the German workers that vacuoles are present post-mortem in the livers of animals dying from acute anoxia was confirmed as follows. Twelve rats were killed by placing them, three at a time, in a small sealed chamber containing soda lime. They died of anoxia at times ranging from 20 to 40 min. Liver samples removed post-mortem showed vacuoles in all cases. Six rats killed with coal gas also showed vacuoles post-mortem. In these experiments, as also in the decompression ones, as a result of anoxia the animals over-

breathed and became acapnic. But it was easy to show that acapnia is not a necessary factor in vacuole formation because six animals placed in the sealed chamber without soda lime, and therefore dying of asphyxia, also showed vacuoles post-mortem. In all these animals the liver was very congested post-mortem.

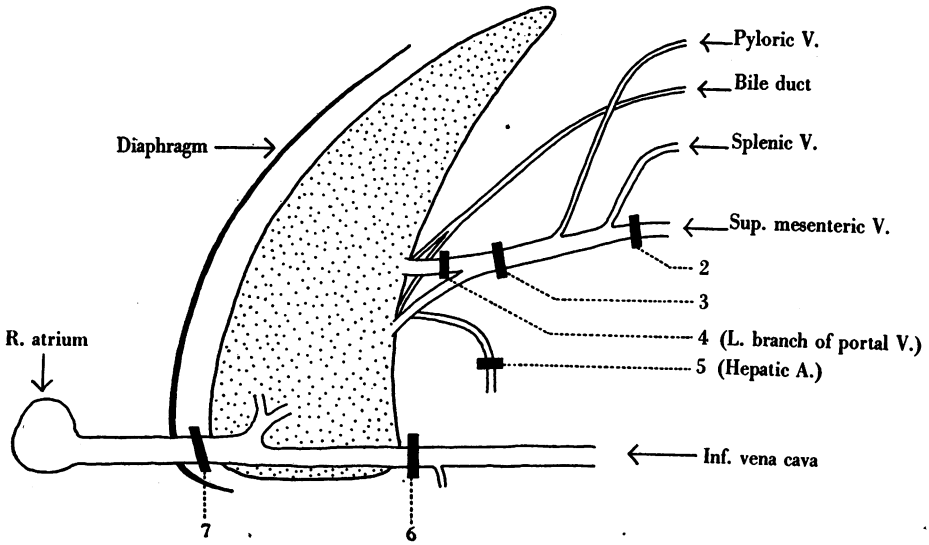
In seeking to relate anoxic death to vacuolation, the first and most obvious suggestion is that the vacuolation represents an early degenerative or necrotic change in the cell cytoplasm resulting from a deficiency of metabolic oxygen. But it was found that slices or fragments of liver removed from a living animal, mounted in blood or plasma and kept at 37° C. with exclusion of air, do not become vacuolated, although the conditions become highly anoxic before the cells eventually die. Similarly, of course, vacuolation is not a feature in ordinary post-mortem liver autolysis. Further, as will be shown later, under certain experimental conditions vacuoles can develop fully in so short a time as 30 sec. So it seems unlikely that vacuolation is a necrotic change in the cytoplasm resulting from liver-cell anoxia; the anoxia must act less directly, perhaps by setting up some nervous or hormonal influence on the liver, perhaps by producing changes in the hepatic circulation. Here, the liver congestion already noted offered a clue. Since there are no venous valves between the right auricle and the liver sinusoids, any rise of venous blood pressure is transmitted back to the sinusoids and, as the portal input pressure is normally very low (5–10 cm. H_2O), the pressure in the sinusoids is to a large extent determined by the general venous pressure. Hence the commonest cause of liver congestion is a rise of venous pressure. Now it is well known that anoxia causes a considerable rise of venous pressure, and in all the experiments so far described a general venous congestion had been found at autopsy. So a hypothesis could be formulated that anoxia causes a rise of venous pressure which is transmitted to the liver sinusoids, and the raised hydrostatic pressure in the sinusoids forces fluid from the blood into the liver cells. This possibility was now investigated experimentally.

(2) *Experimental congestion of the liver*

The vascular connexions of the rat's liver are shown semi-diagrammatically in Text-fig. 1, which also indicates by numerals the points at which vessels were ligated or cannulated in the experiments to be described. Under anaesthesia the abdomen was opened and the vena cava ligated at 7. This caused great congestion of the liver, and, of course, blood stasis and anoxia also. Samples removed 10 min. later showed marked vacuolation. In this experiment the blood which entered and distended the liver presumably came from the portal vein and hepatic artery, but it was also possible that some of it came from the vena cava. The pressure in the abdominal vena cava rises considerably, and it is conceivable that it might even come to exceed the portal pressure and so set up

a reverse flow through the liver. Such an influx of blood from the vena cava would raise various possibilities, like the entry of large quantities of adrenaline from the suprarenal veins; it was therefore desirable to exclude this factor. In one experiment ligature 6 was first tied, then ligature 7; in another, ligatures 3 and 5 were first tied, then ligature 7. In both cases the liver swelled and congested; in the first case the blood came from the hepatic artery and portal vein, in the second from the vena cava. Both livers became equally vacuolated, so the source of the distending blood is unimportant.

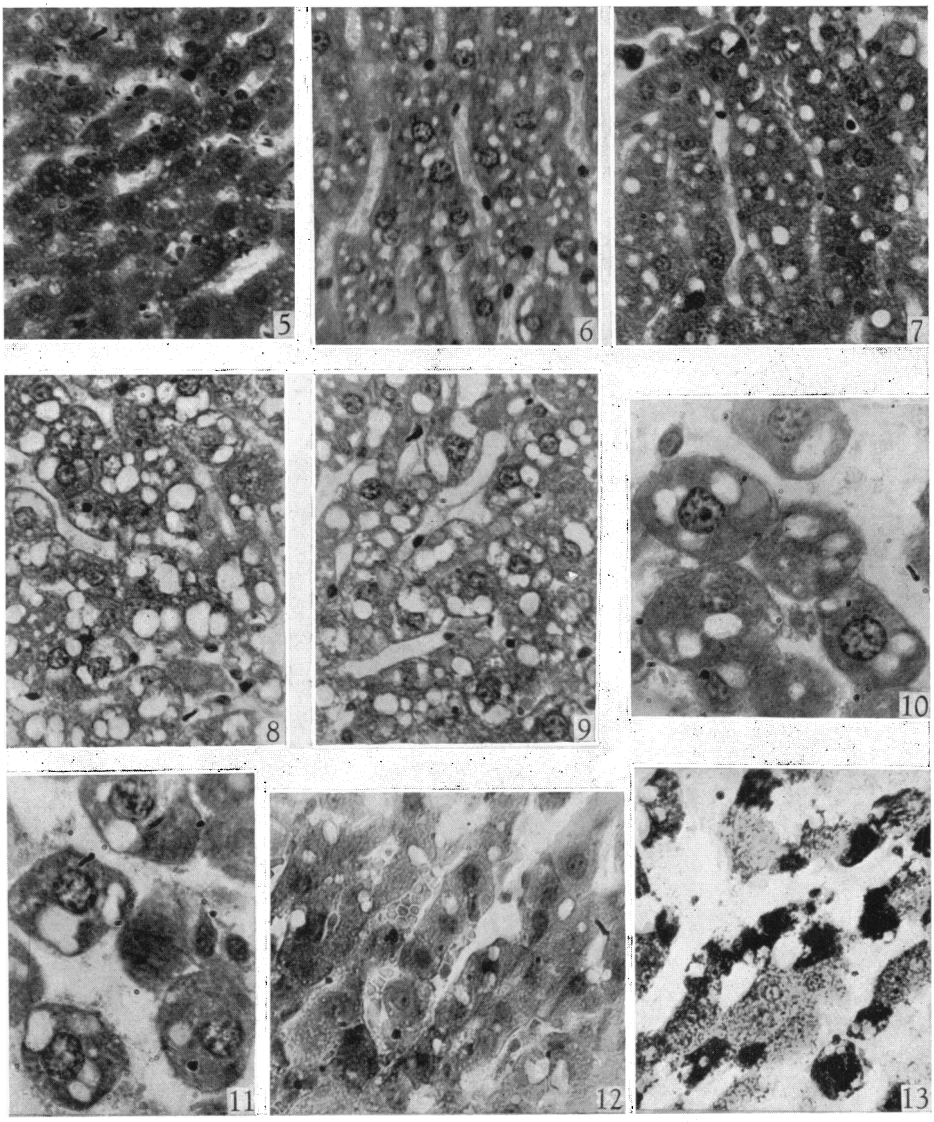
A control experiment to exclude the effects of blood stasis was now performed as follows. Ligatures were loosely placed in positions 6, 5, 3 and 7, and



Text-fig. 1. Diagram of the rat's liver showing vascular connexions. The numbers indicate points of ligature or cannulation referred to in the text.

then quickly tied in that order. This completely arrested the liver blood flow, but produced no congestion. Samples removed at intervals up to 2 hr. showed no trace of vacuoles. Comparing this experiment with the previous ones, in both there was complete blood stasis and therefore severe liver anoxia; the only difference was that in the one case the intrasinusoidal blood pressure was approximately zero while in the other it was greater than normal. The experiments described in this section were repeated three times with the same results, and the conclusion was that liver anoxia (or, more strictly, blood stasis) plus raised intrahepatic blood pressure produces vacuoles, whereas liver anoxia with zero intrahepatic pressure does not.

In these experiments the liver was still in nervous connexion, and for a time in hormonal connexion with the rest of the body. It was therefore desirable to carry the isolation of the liver to completion.



Photomicrographs of sections of rats' livers showing watery vacuolation.

Figs. 5–11. Copper-formol fixation, paraffin sections, haematoxylin and eosin.

Fig. 5. Earliest stage of vacuolation, ×285.

Figs. 6, 7. Moderate vacuolation, ×285.

Figs. 8, 9. Extreme vacuolation, × 285.

Figs. 10, 11. Higher magnification to show typical shapes of vacuoles, ×600.

Fig. 12. Frozen section, haematoxylin and Sudan IV, ×285. The vacuoles do not stain.

Fig. 13. Carnoy fixation, paraffin section, haematoxylin and Best's carmine, ×285. Many of the cells are full of glycogen. The vacuoles do not stain.

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(3) *Production of vacuoles in the isolated liver*

Under anaesthesia, a rat was bled out from the femoral vessels, the abdomen was opened and ligatures were tied at 3, 5 and 6. The chest was now opened, and a cannula, inserted into the inferior vena cava through the right auricle, was tied in at 7. The liver, with cannula, was then entirely removed from the body (for details see Trowell, 1942). In the liver there was now complete stasis, anoxia and zero blood pressure; a sample removed at this time showed no vacuoles. The cannula was now attached to a reservoir of heparinized rat's blood, and the liver was distended with a pressure of 20 cm. H_2O. Samples removed 5, 10 and 30 min. later showed great vacuolation. Similar results were obtained if the liver was distended with normal saline and also if saline was perfused through the liver at 20 cm. H_2O pressure so as to remove all the blood. It was thus clear that vacuolation resulted from intrinsic conditions in the liver itself.

By removing samples at successive intervals of time after applying the distending pressure, the development of the vacuoles could be followed histologically. It was found that they first appear as small vacuoles 2–4 μ in diameter; vacuoles less than 2 μ were never seen, so they appear to originate quite abruptly at this size and not by slow growth from minute submicroscopic vacuoles (Pl. 1, fig. 5). They increase in size mainly by individual growth and to some extent by confluence (Pl. 1, figs. 6–9). The region of the lobule in which they first appear is variable; sometimes it is the centre, sometimes the mid-zone and occasionally the periphery. As the vacuoles increase in size, so the zone of vacuolation extends and ultimately covers the whole lobule. The speed with which the vacuoles develop can be seen from the results listed in Table 2. With a distension pressure of about 20 cm. H_2O the vacuoles are fully developed

TABLE 2. Vascular distension of the isolated liver (rat). The degree of vacuolation found in samples removed at various times after the application of various distending pressures. Each horizontal line represents a single experiment on one animal

0 = no vacuoles.
+ = small vacuoles in some cells.
+ + = medium-sized vacuoles in the majority of cells.
+ + + = large vacuoles in all cells.

Distending pressure (cm. H_2O)	Time after distension										
	5 sec.	10 sec.	15 sec.	30 sec.	1 min.	3 min.	5 min.	8 min.	12 min.	22 min.	42 min.
Saline, 16	—	—	0	+ +	—	—	—	—	—	+ +	—
Saline, 19	—	—	—	+ +	—	+ +	—	—	+ + +	—	—
Saline, 20	+	—	—	+ + +	—	+ + +	—	+ + +	—	—	—
Saline, 25	0	—	0	+ +	+ +	—	+ + +	—	—	—	—
Saline, 3·5–4·0	—	—	—	—	0	—	—	—	+ +	—	+ + +
Saline, 4·0	—	—	—	—	—	—	—	0	—	+ +	+ +
Blood, 3·5–4·5	—	—	—	—	0	—	—	—	+ +	—	+ + +
Blood, 5·0	—	—	—	—	0	0	+	+	+	—	+ +
Saline, 3·0–3·5	—	—	—	—	0	—	—	0	—	0	—
Blood, 3·0–3·5	—	—	—	—	—	0	—	0	0	—	0

in about 30 sec. and show no further increase with time. When the distension pressure is about 4 cm. H_2O, which is scarcely higher than the normal intrasinusoidal pressure during life, the vacuoles appear only after 5–10 min. and require 40 min. or so for full development; but with a pressure of 3–3·5 cm. H_2O no vacuoles develop at all. The critical level of intrasinusoidal pressure required for vacuole formation was determined more carefully as follows. Starting with 2 cm. H_2O pressure, the pressure was raised in steps of 0·5 up to 5 cm. After each increment, the liver was allowed to stand at that pressure for 20 min., and a sample was then removed to see if vacuolation had occurred at that pressure. In these experiments the animal was bled out, the liver was left in situ and distended by a cannula in the portal vein after ligating at 5, 6 and 7. The pressures were measured from the level of the portal fissure. Three such experiments were performed, and the lowest pressures which produced vacuoles were 4, 4 and 3·5 cm. H_2O respectively. This critical pressure for vacuole formation, 3·5–4 cm. H_2O, corresponds roughly with the normal intrasinusoidal pressure during life. When the distension pressure was 3·5 cm. H_2O, the size of the liver corresponded most closely to its size in the living animal, and McQueen (1929) found the normal intrasinusoidal pressure in the mouse to be about 3 cm. H_2O.

The conclusion is that, in the isolated liver, even with maximal anoxia, vacuoles only develop if the intrasinusoidal pressure is maintained at least at its normal level, and the higher the pressure the more rapidly they develop. It may also be inferred that in the intact animal, vacuolation might occur from liver anoxia even in the absence of congestion, but under these conditions it would take at least 10 min. to develop, whereas if congestion were present it might develop much more quickly.

Perfusion and distension of the liver with histological fixatives (formol, Bouin, Carnoy) at 20 cm. H_2O pressure also produced great vacuolation. This again showed how very rapidly vacuolation can occur, for the vacuoles must have developed before the cells were fixed and fixation must have been very rapid; with Carnoy's fluid the liver became quite hard within a minute. These experiments also proved that the vacuoles develop while the distending pressure is actually applied, for they might have been due to a sudden entry of fluid when the pressure on the cells was released.

(4) *Respective roles of anoxia and congestion*

In this paper the term liver 'congestion' is used to mean any condition in which the intrasinusoidal blood pressure is greater than normal. It has just been shown that, in the presence of the extreme anoxia resulting from complete blood stasis, vacuolation occurs even in the absence of congestion, while if congestion is present as well the process is greatly accelerated. But in the whole animal, during life, anoxia can never be so severe as this and it is questionable

if it can ever be severe enough to cause vacuolation in the absence of congestion. Further, in all the experiments so far described anoxia has been present of necessity, and it must be inquired whether congestion alone, in the absence of anoxia, can cause vacuoles. A separation of these two factors, anoxia and congestion, was attempted as follows.

Anoxia without congestion. The simplest way of achieving this is by bleeding. Previous experiments had shown that in the rat, removal of one-third of the blood volume was about the maximum compatible with life. The blood volume was calculated from the body weight (Donaldson, 1924). Under anaesthesia, after opening the abdomen, one-third of the blood volume was removed from the femoral artery. The liver was observed throughout; it was never congested at any stage; after the bleeding it shrank, and remained so. Samples were removed at intervals, and the results of three experiments are shown in Table 3.

TABLE 3. Anaesthetized rats. The degree of vacuolation found in liver samples removed at various times after haemorrhage

Body weight (g.)	Blood volume (c.c.)	Blood removed (c.c.)	Time after haemorrhage				
			5 min.	15 min.	20 min.	30 min.	45 min.
190	12·0	4·0	—	0	+ +	—	—
130	8·4	2·7	0	+	—	+ +	—
130	8·4	2·5	0	0	—	+	+ +

Vacuolation appeared after 20 min. or so but it never became very marked. The vacuoles remained relatively small, not more than 2 or 3 per cell, and were scattered throughout the lobule. These experiments showed that in the living animal anoxia alone, without congestion, can cause vacuolation, but the vacuolation is rather slight and only develops slowly. Anoxia without congestion was also produced by tying off a part of the blood supply to the liver. Neither ligature of the hepatic artery alone nor ligature of the portal vein alone caused any vacuolation within 30 min. When, however, the vessels were ligated at 2 and 5 (Text-fig. 1), so that the liver received blood only from the pyloric and splenic veins, some vacuolation was present after 30 min. These experiments confirm the view that, in the absence of congestion, only very severe anoxia will cause vacuolation, and even then only to a minor extent.

Congestion without anoxia. The only way to produce liver congestion, without at the same time causing anoxia, is to increase the portal input pressure. But, in so doing, no interruption of the blood flow is allowable, for quite a transitory anoxia might have some effect. Consequently, insertion of cannulae and artificial perfusion are not feasible, and in any case it is not possible to maintain a normal blood flow through the liver by perfusion at normal pressures. So other means had to be sought. In the rat the portal vein, as it approaches the liver, divides into right and left main branches, and these divide further to supply the individual lobes. The right branch supplies two lobes which together

comprise about one-third of the liver, while the left branch supplies the remaining five lobes. When the left branch was tied (4, Text-fig. 1) the whole of the portal blood had to pass through the two lobes. These lobes swelled somewhat, and the main portal vein distended to about twice its normal diameter. There was evidently some rise of portal pressure and some congestion of the two lobes, but the congestion was not great. This experiment was performed twice, but no vacuoles were found after 30 min. of such congestion. In a further attempt to produce congestion, 6 c.c. of heparinized rat's blood were slowly injected into the femoral vein of an anaesthetized rat whose calculated blood volume was 14 c.c. This resulted in great distension of the inferior vena cava and moderate distension of the liver. The liver congestion persisted for at least 45 min., and a sample taken at that time showed no vacuoles. In another experiment 5 c.c. of normal saline were injected intravenously into a rat whose blood volume was 11 c.c., and no vacuolation occurred in 35 min. These experiments showed that congestion (to the extent achieved), in the absence of anoxia, does not cause vacuolation. The congestion, however, was less than that which can be achieved by venous obstruction, and it remains possible that more extreme congestion might have had some effect.

(5) *The mechanism of vacuolation*

It is concluded from the foregoing experiments that two factors are necessary for vacuole formation—anoxia and a maintained intrasinusoidal blood pressure. Raised intrasinusoidal pressure (congestion) is an additional augmenting factor which is probably only effective in the presence of anoxia. These are precisely the factors which, in other parts of the body, have been shown to be responsible for the oedema of cardiac failure (Bolton, 1910). This parallelism suggests that, in the case of the liver, anoxia increases the permeability of the sinusoid walls so permitting an increased exudation of tissue fluid. Whereas in other tissues this fluid would accumulate in the tissue spaces, producing oedema, in the liver there is no distensible tissue space between the sinusoid wall and the liver cells, and the fluid therefore passes directly into the liver cells where it accumulates in the form of vacuoles. In other words the vacuoles represent an 'intracellular oedema' of the liver. Evidence for this is as follows.

The vacuoles must represent a watery phase separated by a 'precipitation membrane' from the rest of the cytoplasm which is probably a very viscous colloidal sol. There seem to be three possible ways in which such a watery phase might originate. First, by a physical change in the colloidal state of the cell cytoplasm whereby a watery phase separates out from the colloidal phase. Such changes are known to occur in protoplasm as a result of mechanical injury, pH changes, etc. (Gray, 1931). Secondly, by the loss of some solid constituent, such as glycogen or protein, leaving behind an excess of water which segregates into vacuoles. Thirdly, by an entry of water into the cell from

outside. Now in the first two cases there would be no increase in cell volume, whereas in the third case the cell volume would increase proportionally to the vacuolation. When sections from the same liver before and after vacuolation are compared, it is apparent that the vacuolated cells are larger than the original normal cells. In two experiments the average cell size, before and after vacuolation, was measured as follows. In a section of the normal liver, an area comprising about fifty cells and lying about midway between the centre and periphery of a lobule was selected. The outlines of the sinusoids and nuclei were drawn on paper using a camera lucida. The sinusoids were then cut out, the remaining paper was weighed and the weight divided by the number of nuclei gave the average area per cell, measured as mg. of paper. Similar measurements were made on a comparable area in a section of the same liver after vacuolation. The results are shown in Table 4. During vacuolation the average cell area increased by 21 and 31%. Assuming the cells to be cubical the volume increases would be 34 and 51% respectively. There is therefore no doubt that water enters the cells to form the vacuoles.

TABLE 4. The average cell area in sections of normal and vacuolated liver. Areas measured in arbitrary units (mg. paper). Two experiments on rats

	Total area of cells	No. of nuclei	Area per cell	% increase in cell area
Normal	594	50	11·9	
After vacuolation	719	50	14·4	21
Normal	613	47	13·0	
After vacuolation	649	38	17·1	31

The obvious supposition is that anoxia causes water to enter the cells from the blood. But there is an alternative possibility. Water must be passing slowly through the cells all the time, entering from the blood and leaving in the bile and also probably in the lymph. So anoxia might act by arresting this normal outflow of water from the cells. This explanation can be disproved as follows. In the dog, the known normal rates of bile flow and lymph flow from the liver amount, together, to less than 1% of the liver volume per hour; the figure for the rat is not likely to be greatly different. So if the bile and lymph flows were completely arrested the cells could only swell at the rate of 1% of their volume per hour; whereas it has been shown that the actual rate of swelling may be 50% in 30 sec. Further, if this explanation were correct, it should be possible to produce vacuoles by ligating the bile duct and lymphatics. This experiment was performed. Two bull-dog clips were applied to the lesser omentum so as to clamp all the tissue emerging from the portal fissure, with the exception of the portal vein. The bile duct was obviously occluded and subsequent histological sections showed the portal tract lymphatics widely distended, indicating that the lymphatic outflow had been satisfactorily obstructed. Two such experiments were performed, and no vacuoles were

found after obstruction for 1 hr. It must be concluded that anoxia acts by increasing the entry of water into the cells from the blood.

The next problem is to determine the force which actually causes water to shift from the blood into the cells. The first possibility is hydrostatic pressure, the difference between the intrasinusoidal blood pressure and the pressure in the cell interior. A second possibility is osmotic pressure; anoxia might cause an increase in the osmotic pressure of the cell contents, and the cells would absorb water. A third possibility is imbibition of water by the cell proteins, but this is unlikely as the water would be bound to the protein and not free to collect in vacuoles.

Now it can be shown that osmotic absorption of water does not lead to vacuolation. If small fragments of liver are mounted in distilled water, the process of osmotic swelling can be followed under the microscope. The nucleus swells at once but later shrinks and becomes pyknotic. The cytoplasm swells only very slowly over a period of several hours. There is a progressive 'thinning' of the cytoplasm which may ultimately come to have a rather frothy appearance, and this appears to be due to swelling of the granules, but discrete vacuoles never appear even after many hours. Further, if osmotic forces are involved, distension of the liver with strongly hypertonic solutions should not cause vacuolation, rather water should be withdrawn from the cells. To test this, an isolated liver was first perfused and then distended at 6 cm. H_2O pressure with 50% sucrose; another was similarly treated with 3% NaCl. In both cases vacuolation occurred just as if normal saline had been used. Samples removed 5 min. after distension showed marked and normal vacuolation, but samples removed at 15 min. and later showed great shrinking of the cells and disappearance of nuclei, though the vacuoles remained. Evidently vacuolation first occurred in the ordinary way, and this was followed by osmotic shrinkage and cell necrosis.

By a process of exclusion it seems that the positive hydrostatic pressure in the sinusoids must be the force which actually causes the water to pass into the cells. The fact that there is a critical level of intrasinusoidal pressure below which vacuolation fails to occur and that raising the pressure accelerates vacuolation strongly supports this conclusion. The question whether, on theoretical grounds, a positive pressure in the sinusoids might be expected to force water into the cells raises very complex mechanical problems concerning the internal architecture of the liver which it is not profitable to discuss here.

The essential cause of vacuolation is not a rise of intrasinusoidal pressure, but a change in the permeability of whatever membranes separate the blood from the liver-cell cytoplasm, a change which allows a more rapid transudation of water even at normal pressures. In the normal liver there must be a net filtration force which tends to drive fluid from the blood into the cells, but actual passage of fluid is opposed by the resistance offered by the intervening

membranes, and this resistance is maintained by oxygen. The 'permeability' in question is not that of an inert membrane, it involves forces such as electro-osmosis which are dependent on the vital activity of the cells.

(6) *Vacuolation by distension of the bile canaliculi*

Having shown that, in the anoxic liver, a positive pressure in the sinusoids forces fluid into the cells, it was of interest to see if a positive pressure in the bile canaliculi would do the same. In an isolated liver (bled out) the bile duct was cannulated and distended with saline at a pressure of 25 cm. H_2O. A sample removed after 4 min. showed a few vacuoles just appearing, while a sample at 10 min. showed marked vacuolation. The vacuoles themselves were exactly the same as those seen previously, but their distribution was somewhat different. With bile-duct distension, the vacuoles first appear in the most peripheral cells of the lobules and vacuolation then progresses towards the centre of the lobule in a very regular manner. It seems as if each cell becomes fully vacuolated before the next one is affected at all, and so there is always quite a clear-cut demarcation between the vacuolated zone and the rest of the lobule. After 10 min. the advancing edge of vacuolation had reached about halfway to the centre of the lobule; after 30 min. it had nearly reached the centre. Histologically, the intercellular bile canaliculi were not greatly dilated; evidently they collapse when the pressure is removed, but the vacuoles do not. In another experiment, the pressure was raised in steps, 4·5, 6·5, 10·5, 22 cm. H_2O, at 15 min. intervals. Samples removed at the end of each interval showed that vacuoles did not appear until the pressure was 10·5 cm. H_2O. The critical pressure for vacuolation is therefore higher when the fluid enters from the bile canaliculi than when it enters from the sinusoids, and the speed of vacuolation at comparable pressures is much slower. When the bile duct was similarly distended in a living anaesthetized animal no vacuoles were produced, even after 35 min. distension with a pressure of 25 cm. H_2O. In two experiments the bile duct was tied in the living animal but no vacuoles were produced in 2 hr. It is evident, therefore, that vacuolation from biliary distension, like that from vascular distension, only occurs in the presence of anoxia. The permeability of both sides of the liver-cell is similarly affected by anoxia, but the threshold pressure for water entry differs on the two sides.

(7) *The relation of the liver cells to the blood stream*

The anatomical relation of the liver cells to the blood and lymph is to some extent peculiar, and it will be necessary to define the position before discussing the effects of anoxia on permeability. There has been much discussion as to whether there is a complete endothelial sinusoid wall at all, and, if so, whether there is a space containing tissue fluid between it and the liver cells. The available evidence strongly supports the following view. The sinusoids are

lined by a complete endothelial wall. Between the endothelium and the liver cells is a thin film of protein matrix containing scattered reticulin fibres. The terms 'tissue space' and 'tissue fluid' are often wrongly conceived. There are no empty spaces containing free fluid in any normal tissue. The intercellular spaces are filled with a gelatinous protein matrix, and it is the fluid phase of this colloidal matrix which constitutes the tissue fluid. In the liver, therefore, the thin film of protein matrix between the sinusoid wall and the liver cells constitutes a 'tissue space' and contains tissue fluid. There are no lymphatics in this space; the nearest lymphatics are in the portal tracts at the periphery of the lobule, and the tissue fluid has to seep to this region before being picked up. The tissue space in the liver differs markedly from that in other organs in that it cannot be distended by accumulation of tissue fluid. Procedures calculated to cause accumulation of tissue fluid, such as tying the lymphatics and raising the intrasinusoidal pressure (as in the experiments reported in §§ 2, 3 and 5) do not cause any distension of this space; that is, the endothelial wall does not lift off from the liver cells. It seems that the reticulin fibres are firmly adherent to the liver cells on the one side and to the endothelium on the other, thus binding the two together. The sinusoid wall can only be seen as a distinct entity, separate from the liver cells, at certain stages of liver necrosis when the cells shrink and break away from the endothelium, leaving a gap between. When, as a result of increased permeability of the sinusoids, tissue fluid formation is increased, the tissue space cannot distend to accommodate it, so the hydrostatic pressure in the tissue space rises, and tissue fluid is forced into the cells, producing vacuoles. Instead of ordinary intercellular oedema there results what might be termed 'intracellular oedema'.

The total barrier between the blood and the liver-cell cytoplasm comprises the endothelial sinusoid wall plus the liver-cell wall, and the question arises as to which of these is affected by anoxia. Probably both are. Theoretically, if the permeability of the sinusoid wall (only) were increased by anoxia the liver lymph flow should increase, while if the permeability of the liver-cell wall (only) were affected, the lymph flow would not increase. Unfortunately, there is no direct method of measuring normal liver lymph flow. Starling (1894) obtained indirect evidence that in experimental venous congestion the lymph flow from the liver greatly increases, but his experiments involved both congestion and anoxia, and the effect of anoxia alone on the lymph flow from the liver is not known. In the case of capillaries generally, it is known that anoxia increases permeability, and the same might be expected to apply to the liver sinusoids. The experiments in § 6 indicate that anoxia does increase the permeability of the liver-cell wall, at any rate that part of the wall which faces the bile canaliculus. The question must, therefore, be left open, but it seems likely that both the sinusoid wall and the liver-cell wall are affected by anoxia.

It has been shown that, under anoxial conditions, water and also probably plasma protein pass from the blood into the liver cells. The sinusoid wall must, normally, be almost completely permeable to plasma protein, otherwise the osmotic pressure of the protein would oppose the 3–4 cm. H_2O hydrostatic blood pressure, and no filtration would occur. It was of interest to explore further the permeability of the blood/liver-cell barrier. It was evident that sucrose passed freely into the cells, otherwise vacuolation would have been prevented by the high osmotic pressure set up by 50% sucrose. The permeability to haemoglobin was investigated by distending an isolated liver with an 8% solution of haemoglobin (B.D.H.) in normal saline. The liver was first perfused with the solution to remove the blood and then distended at 18 cm. H_2O pressure for 10 min. Histological sections showed vacuoles, most of which contained a homogeneous material which stained to the same depth and colour as the material in the sinusoids. There is no specific staining reaction for haemoglobin (the benzidine-H_2O_2 method fails at this concentration); the nearest approximation is the bright red staining with eosin. The best results were obtained with eosin and pyrrhol blue, which stained the cytoplasm and nuclei dark blue and the haemoglobin in the sinusoids and vacuoles red. Giemsa staining gave the cytoplasm pale purple and the haemoglobin pink. These results indicate that haemoglobin passes freely into the vacuoles.

Colloidal graphite suspensions were tried next. Ordinary Indian ink is unsuitable because the particles aggregate and block the small vessels. Messrs G. Acheson kindly made for me a special graphite suspension in which the majority of the particles were 0·3 μ. This suspension was stable in the presence of blood and also when perfused through the liver. Two isolated livers were perfused with this suspension and then distended at a pressure of 18 cm. H_2O for 15 min. Histological sections showed graphite particles in the sinusoids, lying in a layer against the endothelium but not filling the whole width of the sinusoid. Normal vacuoles were present but no graphite particles were ever seen within them.

When a liver is injected with warm carmine-gelatin by the ordinary method used for the demonstration of blood vessels, vacuoles are produced and the carmine-gelatin enters the vacuoles quite freely. In sections, the vacuoles are seen to contain carmine-gelatin of the same colour and appearance as that in the sinusoids. It will be recalled that Schafer (1902) claimed that carmine-gelatin injection of the liver revealed networks of intracellular blood canaliculi communicating directly with the sinusoids. This led to the belief that the sinusoids had no endothelial wall, the liver cells being in direct contact with the blood. Schafer's work was extended by Herring & Simpson (1906) who, perfusing the portal vein with carmine-gelatin at pressures of 20–40 mm. Hg, with no obstruction to the venous outflow, were able to inject these so-called canaliculi in the rat, rabbit, guinea-pig, cat and dog. They supported Schafer's

view that the intracellular carmine represented the injection of a network of blood canaliculi, though they stated that 'the injection frequently has the appearance of rounded or irregularly shaped accumulations, as if in vacuoles of the cytoplasm'. The figures of injected cells given by Herring & Simpson and by Schafer (1902, 1929) do not support their conclusions; they show only independent droplets of carmine, which, though they might sometimes be irregular in shape, were not connected into networks. The carmine-injected livers of rat, rabbit, mouse and guinea-pig which I have examined have shown only round or somewhat irregular droplets of carmine, never networks or any connexion with the sinusoids.

Most histologists have regarded Schafer's canaliculi as an artifact due to rupture of the sinusoid and liver-cell walls by the injection pressure, but Schafer (1929) maintained his original view. It seems more likely that the appearances simply represent carmine-gelatin present in vacuoles such as are here described. Presumably, in anoxia the blood/liver-cell barrier is permeable to carmine-gelatin which enters the cells to form vacuoles, just as saline and plasma have been shown to do. Alternatively, it may be suggested that Schafer was right and that all the vacuoles described in this paper are simply his intracellular canaliculi opened up by distension. Against this interpretation is the fact that $0{\cdot}3\,\mu$ graphite particles do not enter, that even with high distension pressure the vacuoles do not appear at once, and that the vacuoles do not disappear when the pressure is released.

It is concluded that in anoxia the blood/liver-cell barrier is permeable to protein (plasma protein, haemoglobin, gelatin) and to colloidal carmine but not to particles of size $0{\cdot}3\,\mu$.

(8) *Development of vacuoles post-mortem*

In death from acute asphyxia, both in animals and man, the classical autopsy finding is systemic venous congestion, indicating a considerable rise of venous blood pressure before death.

In rats killed by anoxia or asphyxia it was noted that the liver and great veins were not only distended by blood but distended under some pressure, for, when the inferior vena cava was opened, blood almost spurted out, and the liver quickly deflated. This suggested that, in animals dying of asphyxia or anoxia, the intrasinusoidal pressure did not fall to zero at death, but maintained a positive value for some time post-mortem. Now if the post-mortem pressure were more than 3·5 cm. H_2O, and if it were maintained for 10 min. or longer, then, on the basis of the findings in the isolated liver, vacuolation ought to occur during the post-mortem period, for the liver is then highly anoxic. So it was possible that the vacuoles found post-mortem in animals dead from asphyxia or anoxia had actually developed during the period of post-mortem

standing, before the autopsy was made, and not during life. This was investigated as follows. In anaesthetized animals the abdomen was opened, and the animal was then killed by sudden asphyxia. Liver samples were removed at the moment of death (i.e. when the heart stopped) and at intervals thereafter. The liver samples were removed by the method of ligaturing previously described so that no blood escaped from the congested liver. Various methods of asphyxia were used and the results are given in Table 5. These experiments

TABLE 5. The degree of vacuolation found in liver samples removed at various times post-mortem in anaesthetized rats killed by sudden asphyxia

Method of killing	Time post-mortem						
	0	2 min.	4 min.	8 min.	15 min.	20 min.	30 min.
Trachea tied	0	—	0	—	—	+ + +	—
Trachea tied	0	0	—	+	—	—	+ + +
Chest opened	0	0	—	+ +	+ + +	—	+ + +
Coal gas	0	0	+ +	+ + +	—	—	—

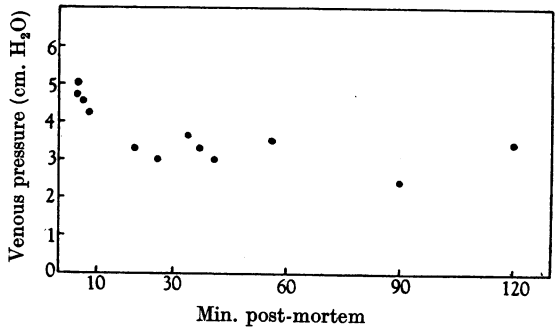
showed that when animals die rapidly from acute asphyxia, no vacuoles form during life, but they develop after about 5 min. post-mortem. If, on the other hand, the animal is bled out as soon as it dies, no vacuoles develop post-mortem. In these experiments the asphyxia only lasted 3–4 min. before the animal died. The effects of a slower asphyxial death were investigated as follows. In a rat under nembutal anaesthesia the trachea was cannulated and connected to a respiration pump, after which the chest was opened. The artificial respiration was now reduced until the animal was asphyxiated and the animal was kept in a condition of maximal asphyxia, just short of death, for 20 min. A liver sample removed at this time showed some vacuoles. The respiration was then stopped, and after 22 min. post-mortem standing the liver showed extreme vacuolation. In another experiment rats were placed, two at a time, in a sealed chamber containing soda lime. When they had almost reached the point of death from anoxia (40–50 min.) they were killed by stunning and at once bled out. It was observed that the heart was still beating. Six animals killed this way all showed some degree of liver vacuolation. These experiments showed that in death from slow progressive anoxia or asphyxia some vacuolation develops before death, but the extreme vacuolation only develops post-mortem.

It was now evident that the vacuoles found in the original decompression experiments and in the general anoxia experiments (§ 1) were in all probability only formed post-mortem, for the livers were always congested, indicating death from asphyxia or anoxia, and the autopsies were not performed until 5 min. or more after death. The mere fact that the animals died an asphyxial or anoxial death would account for the findings, there is no evidence that decompression or anoxia produced any vacuoles during life, though of course they may have done so. This explanation would account for the lack of

correlation between frequency of vacuolation and the degree of duration of anoxia. It would also account for the vacuoles found in the animals which died instantaneously as a result of decompression from 6·0 to 1·0 atm.

Post-mortem vacuolation was also found consistently in a large series of rats and rabbits (starved) killed by nitrogen or coal gas, and experience suggested the following generalization. In starved animals (rat or rabbit), if at an autopsy performed not sooner than 15 min. from the time of death the liver is found to be congested, then that liver will contain vacuoles.

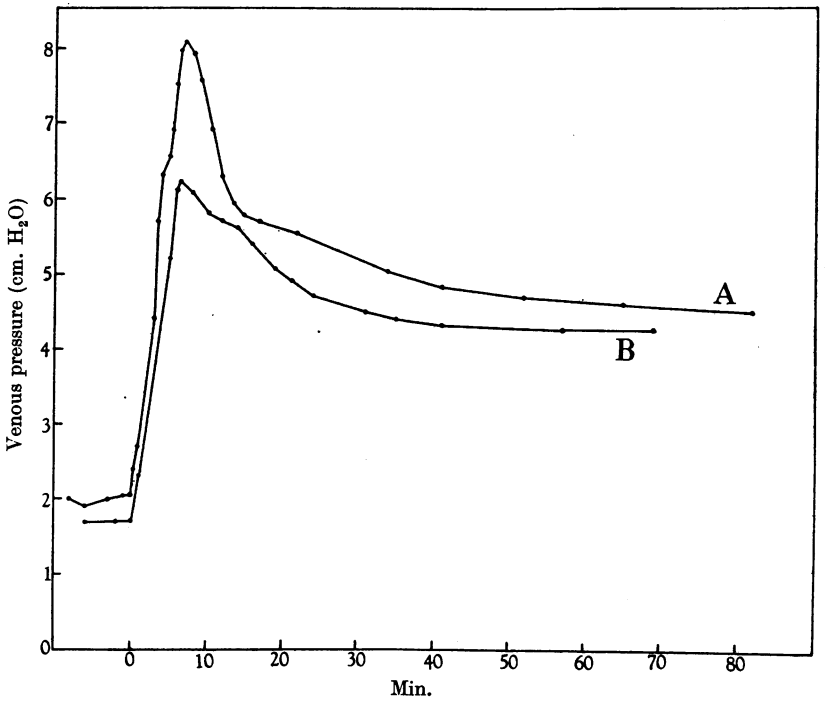
The development of vacuoles during life can only be proved by finding them in liver samples removed during life, or in samples removed post-mortem from animals which were bled out at the time of death. That liver vacuoles do develop during life, in conditions of severe and prolonged general asphyxia or anoxia, has already been shown in this way.



Text-fig. 2. The venous blood pressure at various times post-mortem in rats killed by anoxia. Each point represents a different animal.

The original argument was that, if, after death, a venous pressure higher than 3·5 cm. H_2O were maintained for at least 10 min., then theoretically vacuolation ought to occur during this period. Having shown that such vacuolation does occur after asphyxial death, it now remains to complete the evidence by showing that the post-mortem venous pressure does fulfil these requirements. The venous pressure was measured by inserting a hypodermic needle, attached to a saline manometer, into the inferior vena cava so that the tip of the needle lay in the intrahepatic part of the vena cava. An ordinary Warburg manometer was used with one limb cut short and connected to the needle. In this way the manometer level could be very rapidly adjusted so as to avoid any entry of saline or withdrawal of blood during equilibration. After insertion, the needle was held firmly in position by a clamp, and at the end of each experiment zero pressure was determined by widely opening the vena cava, leaving the needle undisturbed. In the first experiment twelve rats were killed by anoxia by placing them in a sealed chamber with soda lime. In each animal the venous pressure was measured at a certain time after death, the

times differing in the different animals. The results are shown in Text-fig. 2. The next experiments were performed on anaesthetized rats. After opening the abdomen and allowing the wound edges to coagulate, 200 units of heparin were injected into the femoral vein to prevent subsequent clotting in the venous pressure needle. This needle was now inserted and firmly fixed in position. After recording the normal venous pressure the animal was killed, either by opening the chest or tying the trachea in the neck, and venous pressure readings were made at frequent intervals from the beginning of asphyxia till after death.



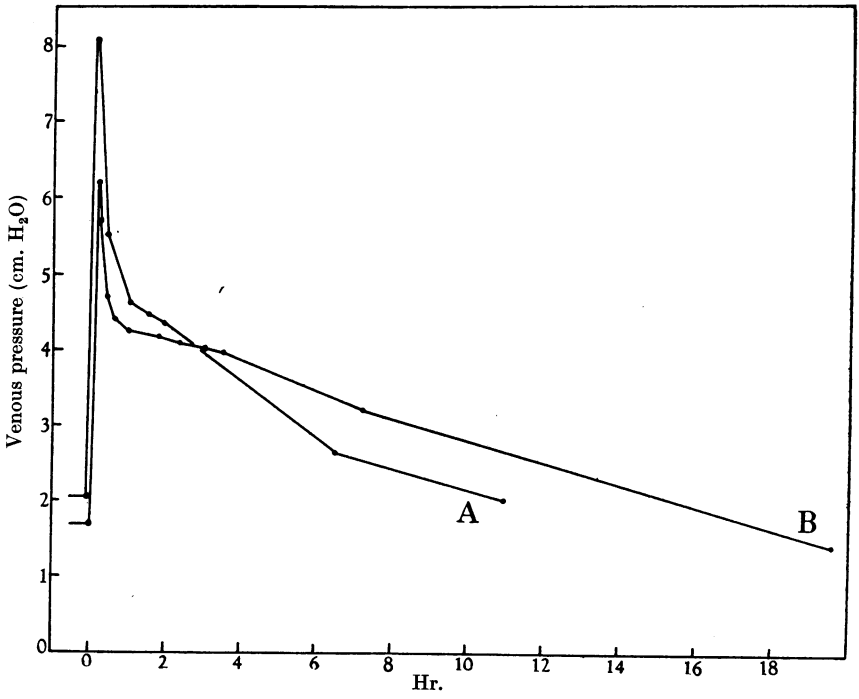
Text-fig. 3. The venous blood pressure before, during and after death in anaesthetized rats killed by sudden asphyxia. At zero time, the trachea tied in Exp. A, the chest opened in Exp. B.

Four experiments were performed and the results of two of them are shown in Text-fig. 3 and on a longer time-scale in Text-fig. 4; the other two gave similar results. It is evident that, after asphyxial death, the intrahepatic venous pressure remained higher than 4 cm. H_2O during the first hour post-mortem and above 3 cm. H_2O for several hours, and so the original prediction is shown to be true.

(9) *Reversibility of vacuolation*

It was discovered that when large (2 cm.^{3}) pieces of vacuolated liver were fixed in formalin, the subsequent sections often showed vacuoles only in the peripheral part of the block and none in the centre. Whereas similar-sized

pieces fixed in a rapidly penetrating fixative, such as Carnoy, showed vacuoles throughout. So, if large pieces of liver are fixed in formalin, and the blocks are trimmed down before embedding, vacuolation may be missed. Fortunately this difficulty was discovered early in the work and it is believed that, except perhaps in some of the decompression experiments, no errors have arisen from this source. The simplest interpretation seemed to be that the vacuoles slowly disappear on standing, and that at the centre of a large block they may have disappeared by the time the slowly penetrating formalin reaches and fixes the



Text-fig. 4. The same as Text-fig. 3 but on a longer time-scale.

cells. It was found, however, that pieces of vacuolated liver allowed to stand at room temperature, either protected from air or immersed in saline, for 5 hr. (two experiments) or 24 hr. (two experiments), showed just as many and as large vacuoles as did pieces of the same liver fixed immediately. So it seems that under ordinary conditions the vacuoles do not disappear on long standing in vitro. The explanation of the formalin results is not clear.

It was thought that by applying an external pressure to the liver it might be possible to reverse the vacuolation and drive the vacuolar fluid back into the blood. An isolated liver was first vacuolated by distending it with saline, and a sample was removed. The liver plus cannula was then placed at the bottom of a tall glass cylinder with the cannula passing through a cork to the exterior. The

cylinder was filled with saline to a height of 20 cm. above the liver. The liver was thus subjected to an external pressure of 20 cm. H_2O, while the vena cava and, theoretically, the sinusoids were in free communication with the outside. After 1 hr. samples showed no reduction of vacuolation as compared with the original sample.

In contrast to this non-reversibility in vitro, it was found that in vivo the vacuoles disappeared fairly quickly when the exciting cause was removed. In an anaesthetized rat the abdomen was opened, and the vena cava was totally obstructed just below the diaphragm. This was done by passing the handle of a teaspoon between the liver and the diaphragm and compressing the vena cava against the vertebral column. After 10 min. obstruction a liver sample was removed and showed considerable vacuolation. The vena cava was now released, and a sample taken 30 min. later, at which time the animal was alive and in good condition, showed that the vacuoles had almost entirely disappeared.

(10) *The relation of vacuolation to liver glycogen*

The original observations on rabbits (unstarved) revealed an inverse correlation between the frequency of vacuolation and the glycogen content of the liver (Table 1). This suggested that, either the vacuoles resulted in some way from the actual breakdown of liver glycogen, or else a high glycogen content protected the liver from vacuolation. The evidence against the former explanation is as follows. Analysis of the observations on 200 rabbits showed that the glycogen content of the liver was quite unrelated to the degree or the duration of the anoxia to which the animals were exposed before death. The evidence, such as it was, indicated that anoxia did not cause any great breakdown of liver glycogen. Haist & Hamilton (1944) found that, in the case of rats fed on glucose, exposure to low atmospheric pressure for 4 hr. resulted in a decrease of liver glycogen, but Evans (1934) found in fasting rats that exposure to low pressure caused an increase of liver glycogen. Liver samples removed from rabbits at the time of death and at intervals thereafter showed (contrary to expectation) that no significant fall in the histological level of liver glycogen occurs during 2 hr. post-mortem standing. Conclusive proof that, in the rat, vacuolation is not due to the breakdown of liver glycogen was obtained as follows. In many of the experiments, already described, in which vacuoles were produced by ligature of the vena cava or by distending the isolated liver, portions of the samples removed were fixed in Carnoy and used for glycogen staining. The sections showed quite clearly that vacuole formation was neither preceded nor accompanied by any change in the histological level of liver glycogen.

Evidence will now be given in favour of the second hypothesis, that a high glycogen content protects the liver from vacuolation. In all the work on the experimental production of vacuoles in rats, starved animals were used, and in

them the liver glycogen was always low. Most of these experiments were repeated several times and vacuolation occurred in all cases under the conditions which have been specified. When, however, these same experiments were performed on unstarved animals, vacuolation often failed to occur, and, when it did fail, the liver glycogen was always found to be high. The following experiments on unstarved rats may be cited. Asphyxial death in a closed chamber produced vacuoles in only seven out of sixteen animals, killing by coal gas in five out of ten. Tying the vena cava above the liver produced no vacuoles in one case and a minor degree of vacuolation in three cases. Saline distension of the isolated liver at 6 cm. H_2O pressure gave good vacuolation (one case), slight vacuolation (two cases) and no vacuolation (one case). In three rats the liver glycogen was artificially raised by feeding a high carbohydrate diet for 24 hr. before the experiment. In all three animals distension of the liver with saline at 6 cm. H_2O pressure for 20 min. failed to produce any significant vacuolation, but further distension at 20 cm. pressure for 7 min. did produce considerable vacuolation. Evidently the threshold pressure for vacuolation is higher when the liver is rich in glycogen. From these experiments it was concluded that a high level of liver glycogen may prevent vacuolation in circumstances under which it would otherwise occur.

According to Kater (1933), in the rabbit the liver lobule fills with glycogen from the centre to the periphery, whereas in the rat it fills from the periphery to the centre. So when the glycogen content is small or moderate the glycogen is confined to the centre of the lobule in the rabbit and the periphery in the rat. Now if the concept that a high glycogen content protects against vacuolation applies not only to the whole liver but also to individual cells, then it would be expected that vacuoles should appear first in the outer part of the lobule in the rabbit and in the central part in the rat. In fact, it was found that in both species vacuoles might first appear in any part of the lobule, but it was, nevertheless, true that the centre of the lobule was the least common site for vacuolation in the rabbit and the periphery in the rat. The issue was complicated by the fact that although in most cases the lobular distribution of glycogen followed Kater's description, in some cases it did not. Thus livers were found in which a moderate amount of glycogen was distributed uniformly through the lobule, while in others glycogen-rich and glycogen-free cells were irregularly scattered throughout the lobule; in this latter type it was observed that vacuoles occurred just as frequently in the glycogen-rich as in the glycogen-free cells. Another complicating factor is that, if the vacuoles are due to anoxia, then the oxygen gradient in the lobule should influence their distribution, favouring their occurrence at the centre of the lobule. It is likely that the lobular distribution of vacuoles in any particular case is determined by a variety of factors: the glycogen gradient, the oxygen gradient, and probably other gradients such as intracellular pressure, tissue tension, etc. The most

that can be said with regard to glycogen is that vacuoles develop more readily in glycogen-poor livers, that they tend to develop first in that part of the lobule which is poorest in glycogen, but a strict correlation between glycogen content and vacuolation in the case of individual cells cannot be shown. It is known that the liver cells increase in volume considerably when they accumulate glycogen and it has been shown that they increase in volume on vacuolation. So it may be that a cell rich in glycogen is already considerably distended and therefore further distension by vacuolation is more difficult. The explanation may, however, be metabolic rather than mechanical. The liver has some power of anaerobic glycolysis, that is it can obtain energy even in the absence of oxygen by breaking down glycogen to lactic acid. In anoxia, therefore, the vital activity of the cells may come to depend on their glycogen store. There is good reason to believe that the proper permeability of cell membranes is maintained by the energy-yielding metabolism of the cell, so it is possible that even in the absence of oxygen the liver cells can preserve a proper permeability provided that sufficient glycogen is available to maintain glycolysis.

DISCUSSION

Vacuoles such as these have not been described in the normal liver, nor are they ordinarily recognized in human pathology. They have, however, been recorded from time to time in animals under various experimental pathological conditions, but their significance has usually been misinterpreted.

The normal liver. Vacuoles, of a sort, have been described in the normal liver. Noel (1923) described 'neutral red' vacuoles in the normal liver-cell of the mouse. They were minute, few in number, situated close to the nucleus and only visible after supravital staining with neutral red. In the rat and rabbit I have been unable to find any vacuoles staining supravitally with neutral red. Noel also described, as a rapid post-mortem change, vesiculation of the mitochondria leading to the appearance of tiny vacuoles, but these, like the neutral red vacuoles, were very minute and much smaller than the smallest of the vacuoles found in anoxia. Kater (1933) described watery vacuolation associated with the deposition of glycogen in many species, including the rat and rabbit. According to him, all liver cells poor in glycogen contain large watery vacuoles; glycogen is first deposited round these vacuoles and as the glycogen accumulates the vacuoles shrink and disappear, to reappear when the glycogen is withdrawn. I have not seen these appearances, that is, vacuoles and deposition of glycogen around vacuoles, in the normal livers of rat, rabbit, guinea-pig or monkey. Nevertheless, his observation that the vacuolation was greatest in the glycogen-poor cells and least in the glycogen-rich cells is in agreement with what has been found here. Kater does not mention how his animals were killed, but if they were killed by some form of anoxia (e.g. coal gas) then those poor in glycogen would certainly exhibit the post-mortem vacuolation which

has been described in this paper, and so it is possible that this is what he was observing. Policard (1934) describes large empty vacuoles present in the liver cells only after meals when the cells are large and distended and says that they are due to absorption of water by the liver during digestive activity. It seems probable that he was simply referring to the glycogen spaces which are normally seen in paraffin sections and which are, of course, a histological artifact. For many years it was believed that the liver-cell contains small secretion vacuoles connected with a fine network of intracellular bile canaliculi (Oppel, 1900), but it is now thought that the appearances were due to staining of parts of the Golgi apparatus and that such vacuoles do not exist (Maximow & Bloom, 1944).

Pathological livers. The watery vacuolation described in this paper seems to have been first observed by Raum (1892). He slowly infused very large volumes of saline into the jugular vein of anaesthetized dogs and killed the animals by tying the trachea about 3 hr. from the start of the infusion. Post-mortem samples of liver showed vacuolation of the cells. He showed that the vacuoles contained neither fat nor glycogen and described them as often ovoid or ellipsoidal rather than spherical and as sometimes indenting the nucleus. He thought they arose from swelling of the fuchsinophil granules (mitochondria), but provided no evidence for this. In one experiment he infused saline for 50 min. and removed a liver sample from the living animal 95 min. later. This sample showed vacuolation. The animal was finally killed after a further 5 hr. and the liver then contained no vacuoles. From this he concluded that the vacuolation was a very transitory phenomenon and he regarded the vacuolation as a 'hydropic infiltration' of the liver cells resulting from hydraemic plethora. He noted that the livers were greatly congested, and there was in addition great circulatory embarrassment, so it would be easy to account for his findings in terms of anoxia and congestion of the liver.

Watery vacuolation has been described in certain types of experimental liver necrosis. Mallory (1901) in a study of liver necrosis in rabbits produced by diphtheria toxin found that watery vacuolation was apparently the first stage in cell necrosis. The vacuoles contained tiny globules or threads of fibrin. It may be mentioned that the anoxic vacuoles described here never contained any fibrin (Weigert staining). Ogilvie (1932) poisoned rabbits with mercuric chloride and after death found that the livers showed congestion, cloudy swelling and sometimes a little necrosis. In three out of sixteen animals the cells showed watery vacuolation, similar to that described here, and this was described as 'hydropic degeneration' of the liver. Drummond & Paton (1904) killed rabbits by injecting very large doses of adrenaline daily until the animal died. Post-mortem they found central degeneration of the liver and in some cases the degenerating cells contained large watery vacuoles. They showed that the vacuoles contained neither fat nor glycogen and believed that they represented an early stage of cell necrosis. All these workers regarded the

vacuolation as a degenerative or necrotic change. This may be true, but from the evidence presented in this paper it seems possible, if not probable, that the vacuolation was simply due to venous congestion of the liver produced by the experiments (Mallory and Ogilvie noted that their livers were congested) or perhaps to the fact that the animals ultimately died of anoxia. It is also possible that the various poisons employed damaged the blood/liver-cell barrier, increasing its permeability just as anoxia does. Pappenheimer & Hawthorne (1936) in 31% of 562 routine human autopsies and in twenty-six of thirty-four still-born human foetuses found watery vacuoles in the liver, but the vacuoles each contained a single spherical inclusion body 1–3 μ in diameter and sometimes rods and filaments as well. The inclusion bodies were acidophilic and resembled virus inclusions. They were unable to correlate the vacuoles with any disease but noted that they were more numerous in congested livers. They found similar vacuoles with inclusions in normal monkeys, guinea-pigs and ferrets, but not in cats, dogs, rats or rabbits, and they were unable to reach any conclusions as to their significance.

Of greater interest is liver necrosis produced experimentally by prolonged anoxia. Here, one would expect vacuolation to occur but apparently this has not been the general experience. Goldschmidt, Ravdin & Lucké (1937) exposed dogs to an atmosphere of 15% oxygen for 2 hr. and examined the liver 4 days later. They found liver necrosis in some of the animals but did not report vacuolation. Martin, Bunting & Loevenhart (1916) exposed rabbits to 5–9% oxygen for a week and found central necrosis, fatty degeneration and also 'serous imbibition' in some of the cells; probably 'serous imbibition' was their interpretation of watery vacuolation. Cameron & Mayes (1930) tied the hepatic artery in rabbits and examined the livers of animals killed 3, 6 and 12 hr. later. Liver necrosis began at 12 hr. but no vacuolation was reported at any stage. Campbell (1927) exposed rabbits and guinea-pigs to atmospheres low in oxygen, the animals dying after several days. At autopsy the livers were greatly congested and showed fatty degeneration. McMichael (1937) tied portal vein branches in the cat and found intense fatty degeneration of the affected part of the liver 24 hr. later. Neither Campbell nor McMichael mention any control fat staining of their material; doubtless this was done, but, if not, the vacuoles they saw in paraffin sections may have been watery ones. Bolton & Barnard (1931) produced chronic liver congestion in the cat by placing a constricting band round the thoracic inferior vena cava. Animals killed 4 and 6 days later showed central necrosis of the liver; it was also mentioned that 'vacuoles appear' in the cells, but whether they were fatty or otherwise was not stated.

The fact that watery vacuolation has not as a rule been found in these experiments on anoxic liver necrosis may perhaps be explained as follows. It has already been shown that vacuolation in vivo only occurs when anoxia is

very severe. It seems likely that in most of these experiments on anoxic necrosis the anoxia was not severe enough to cause vacuolation, but, acting over a period of several days, it was severe enough to cause necrosis. The next point concerns the time-scale of the observations. In most of the experiments the liver was not examined until several days from the beginning of anoxia, whereas the observations described in this paper relate only to the first 1–2 hr. from the onset of anoxia. It has been shown that the vacuolation rapidly disappears when the exciting cause is removed, and it is quite possible that even when the anoxia is maintained the vacuolation may disappear owing to adaptation on the part of the liver. So vacuolation may be only an initial transitory change which the liver rapidly compensates and cures; but there is no direct evidence on this point. Such considerations could explain why watery vacuolation does not (apparently) occur in cases of human chronic venous congestion (nutmeg liver). It is by no means certain, however, that watery vacuolation does not occur in human chronic venous congestion. In such cases the liver cells are greatly vacuolated and although it is generally believed that the vacuoles are fat, it seems desirable that such livers and others showing 'fatty degeneration' should be more carefully investigated with Sudan staining. It may thus be discovered that some of the vacuoles are non-fatty and it may be that watery vacuolation of the liver occurs in human pathology more commonly than has been supposed.

Finally, reference must be made to the production of what appears to be the same watery vacuolation by the feeding of diets deficient in protein. Elman & Heifetz (1941) fed dogs on a low protein diet for 3 weeks and found that the liver cells developed vacuoles which contained neither fat nor glycogen. They believed that the vacuoles were watery and were due to removal of protein from the liver cells. Dean (1942) fed mice on a diet consisting chiefly of sucrose for 2–6 weeks and found similar watery vacuolation of the liver. The vacuoles produced by protein starvation appear, histologically, to be just the same as those produced by anoxia, but it is not at present possible to relate these two sets of findings. It may be that lack of protein in the liver cells or in the blood disturbs the permeability of the blood/liver-cell barrier in the same way that anoxia does.

Addendum. Kritzler (1944) has reported autopsy findings in twenty-seven airmen whose death was attributed to anoxia. In twenty-four cases the liver showed watery vacuolation. In size, shape and numbers the vacuoles were similar to those described in this paper. There was, however, one important difference. In his cases the vacuoles each contained a single eosinophilic inclusion particle apparently the same as those already described in man by Pappenheimer & Hawthorne (1936). Similar vacuoles and inclusions were also found in four out of five fatal cases of carbon monoxide poisoning. In the

animal experiments described in this paper such inclusion particles were never seen, though sometimes the vacuoles were filled with a diffuse protein material. It seems likely that this is a species difference in the protein content of the vacuoles and that the fundamental cause of vacuolation is the same. Kritzler also found similar vacuoles in the cardiac muscle fibres, acinar cells of the pancreas, chromaphobe cells of the pituitary, parietal cells of the stomach and sometimes in the thyroid.

SUMMARY

1. A state of watery vacuolation of the liver is described. Discrete vacuoles, 2–12 μ in diameter and approximately spherical, occur in the liver-cell cytoplasm. They are visible in the living cell and contain neither fat nor glycogen. In sections they usually appear as empty spaces, but sometimes they contain a faintly staining material which appears to be protein. This vacuolation has been observed in the rat, rabbit, guinea-pig and monkey.

2. In the starved rat this vacuolation could be regularly produced by any of the following procedures: (i) severe anoxia or asphyxia of the whole animal maintained for 20 min. or more, (ii) venous congestion of the liver produced by tying the vena cava above the liver, (iii) severe haemorrhage, (iv) tying both the hepatic artery and the superior mesenteric vein, (v) distension of the blood vessels of an isolated liver with blood or saline at a pressure greater than 3·5 cm. H_2O—the higher the pressure, the more rapid the vacuolation.

3. In the isolated (anoxic) liver, when the intrasinusoidal pressure was raised to 4 cm. H_2O vacuolation first appeared after about 10 min. and was fully developed at 40 min. When the pressure was 20 cm. H_2O the vacuolation fully developed in 30 sec. The critical pressure required for vacuolation was 3·5–4·0 cm. H_2O.

4. In the starved rat, raised intrasinusoidal blood pressure without anoxia did not cause vacuolation, neither did ligature of the bile duct and liver lymphatics.

5. It was concluded that two factors are necessary for vacuolation, (i) liver anoxia, and (ii) the intrasinusoidal pressure must be maintained at least at its normal value (3–4 cm. H_2O); if it is greater, then vacuolation occurs more rapidly, but, within physiological limits, raised pressure alone does not cause vacuolation.

6. Evidence is presented that the vacuolation is not a degenerative nor necrotic change, that it is due to the entry of fluid into the liver cells from the blood, and that the entry is due to the hydrostatic pressure in the sinusoids and not to osmotic absorption.

7. The theory is advanced that anoxia by altering the 'permeability' of either the sinusoid wall or the liver-cell wall allows the existing hydrostatic pressure in the sinusoids to force fluid from the blood into the cells. The vacuolation could thus be regarded as an 'intracellular oedema' of the liver.

8. In anoxia, plasma protein, haemoglobin and carmine-gelatin, but not colloidal graphite, could enter the vacuoles from the blood stream.

9. The vacuolation was found to be reversible in vivo.

10. In starved rats, dying from sudden (or prolonged) asphyxia, vacuolation developed during the first 10 min. after death provided that the animals were not bled out. After death from asphyxia there is great venous congestion and it was shown that the intrahepatic venous pressure remains higher than 4 cm. H_2O for at least 10 min; so in these circumstances the intrasinusoidal pressure is sufficiently high to produce vacuolation of the post-mortem anoxic liver.

11. In normal (unstarved) animals, the experiments listed in §§ 2, 3 and 10 above produced vacuoles in only 30–50% of animals; vacuolation failing to occur in those with a high liver glycogen. An inverse correlation between vacuolation and the liver-glycogen level was found. It was shown that vacuolation does not result from the breakdown of liver glycogen and it seemed that the glycogen-rich liver is in some way resistant to vacuolation.

12. Distension of the bile canaliculi with saline produced vacuolation in the anoxic liver but not in the normal liver in vivo. The critical pressure for vacuolation in this case was 10·5 cm. H_2O.

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